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Novel *FH* mutations in families with hereditary leiomyomatosis and renal cell cancer (HLRCC) and patients with isolated type 2 papillary renal cell carcinoma

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ABSTRACT

Background Hereditary leiomyomatosis and renal cell cancer (HLRCC) is an autosomal dominant disorder predisposing humans to cutaneous and uterine leiomyomas; in 20% of affected families, type 2 papillary renal cell cancers (PRCCII) also occur with aggressive course and poor prognosis. HLRCC results from heterozygous germline mutations in the tumour suppressor fumarate hydratase (*FH*) gene.

Methods As part of the French National Cancer Institute (INCa) 'Inherited predispositions to kidney cancer' network, sequence analysis and a functional study of *FH* were preformed in 56 families with clinically proven or suspected HLRCC and in 23 patients with isolated PRCCII (5 familial and 18 sporadic).

Results The study identified 32 different germline *FH* mutations (15 missense, 6 frameshifts, 4 nonsense, 1 deletion/insertion, 5 splice site, and 1 complete deletion) in 40/56 (71.4%) families with proven or suspected HLRCC and in 4/23 (17.4%) probands with PRCCII alone, including 2 sporadic cases. 21 of these were novel and all were demonstrated as deleterious by significant reduction of FH enzymatic activity. In addition, 5 asymptomatic parents in 3 families were confirmed as carrying disease-causing mutations.

Conclusions This study identified and characterised 21 novel *FH* mutations and demonstrated that PRCCII can be the only one manifestation of HLRCC. Due to the incomplete penetrance of HLRCC, the authors propose to extend the *FH* mutation analysis to every patient with PRCCII occurring before 40 years of age or when renal tumour harbours characteristic histologic features, in order to discover previously ignored HLRCC affected families.

INTRODUCTION

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC, OMIM 605839) is an autosomal dominant familial disorder characterised by the development of cutaneous and uterine (fibroid) leiomyomas, renal cell carcinoma (RCC), and rarely uterine leiomyosarcomas.[1 2] HLRCC was previously called multiple cutaneous and uterine leiomyomatosis (MCUL, OMIM 150800), as the association between skin and uterine leiomyomas was described before the discovery of RCC predisposition. Cutaneous leiomyomas occur in 76% of individuals at a mean age of 25 years (range 10–47 years), but 40% of individuals have mild cutaneous manifestations with five or fewer lesions.[3 4] Uterine leiomyomas are present in almost all women with a mean age at diagnosis of 30 years (range 18–52 years).[3–5] RCC occurrence is relatively low (20%, 29/144 families worldwide) and differs between and within families affected by HLRCC.[1 3 4 6–24] RCCs predominantly affect young (<40 years) adults with a mean age at diagnosis of 46 years (range 17–75 years).[25] They are usually solitary, unilateral, and highly aggressive with rapid dissemination. The main histological RCC subtype is type 2 papillary RCC (PRCCII), a variety of renal cancer characterised by large

tumour cells with eosinophilic cytoplasm and pseudostratified nuclei.[17 25 26] Less frequently, collecting duct RCC may also be observed in patients with HLRCC.[4 25]

MCUL/HLRCC is associated with heterozygous germline mutations in the fumarate hydratase (or fumarase, *FH*) gene located at 1q42.3–q43.[2 3 6] Interestingly, homozygous and compound heterozygous *FH* mutations have been first identified in fumarase deficiency, a rare autosomal recessive disorder characterised by neurological impairment and death in the first decade of life (FHD; MIM 136850, OMIM 606812).[27–29] *FH* spans 22 kb, contains 10 exons, and encodes two fumarase isozymes, mitochondrial and cytosolic. The active form of FH is a homotetramer in which three of the four chains combine to form the enzymatic active site.[7 30 31] FH catalyses the conversion of fumarate to malate in the mitochondrial matrix as part of the tricarboxylic acid (TCA) cycle. Thus, FH deficiencies result in chronic accumulation of fumarate and altered concentrations of other TCA intermediates.

Fumarate accumulation has been shown to induce activation of hypoxia inducible factor (HIF) and its target genes.[32] HIF plays a major role in the tissue response to hypoxia by inducing expression of multiple genes involved in cell survival and proliferation. In renal cancers and fibroids from HLRCC patients, α subunits of HIF (HIF1α and 2α) are overexpressed.[32 33] Moreover, mice with inactive *Fh1* in the kidney developed proliferative renal cysts overexpressing HIF α subunits and hypoxia pathway factors.[34] FH alteration induces fumarate accumulation and the production of reactive oxygen species, leading to activation of the HIF α subunits through inhibition of prolyl hydroxylase (PHD2), triggering the VHL dependent degradation of HIF α subunits under normoxia.[35] Therefore, *FH* inactivation seems to be implicated in inappropriate activation of oncogenic hypoxia pathways, similar to the manner in which *VHL* germline mutations result in von Hippel–Lindau disease, the main cause of hereditary clear cell RCC (CCRCC). However, in contrast to the frequent observation of somatic *VHL* mutations in sporadic CCRCCs, to date no *FH* mutations have been detected in sporadic PRCCIIs.[36]

To date, four complete deletions of *FH*, one exon deletion, one exon duplication, and 81 different *FH* germline point mutations have been reported in 144 MCUL/HLRCC families (supplementary table 1).[1 3 4 6–24 37–39] There is no obvious relationship between genotype and the aggressiveness of the disease. All these mutations lead to the loss of FH enzymatic activity; the remaining functional allele is lost in most cutaneous, uterine, and renal tumours arising in patients carrying *FH* germline heterozygous mutations.[6 7] The function of *FH* is then consistent with the function of tumour suppressor genes.[6]

As part of the French National Cancer Institute (INCa) 'Inherited predispositions to kidney cancer' network, we performed the first comprehensive genetic and functional analysis of *FH* in a large series of patients with phenotypes highly suggestive of MCUL/HLRCC disease and in patients with only PRCCII. These analyses enhance our knowledge of the *FH* germline mutational spectrum and expand the group of phenotypic features associated with these *FH* mutations.

PATIENTS AND METHODS

Patient selection

Families were selected for inclusion in the study through two clinical approaches. Dermatology departments identified and recruited 56 families with clinical histories demonstrative (N=44) or suggestive (N=12) of MCUL/HLRCC. We defined a family as clinically affected if at least one member had more than 10 skin lesions clinically compatible with leiomyomas, including a minimum of one lesion histologically confirmed. Patients with single cutaneous leiomyoma, isolated or associated with personal or familial uterine leiomyomas or RCC, were classified as suggestive of potential MCUL/HLRCC. All alive patients had a detailed examination of the skin. Uterine fibroids and renal tumours were documented by history and review of medical records. Renal ultrasound or MRI was performed in all adult patients. A transvaginal ultrasound was also performed in all women who still had a uterus. In addition, urology departments recruited 18 patients with apparent sporadic PRCCII and five patients with familial PRCCII.

All patients were monitored by French physicians, with the exception of six families who were followed by international colleagues (Singapore, Australia, Belgium, Canada, and New Zealand). This study was approved by the Ethical Committee of Le Kremlin-Bicêtre University Hospital, France. All patients had previously provided informed consent for genetic testing and use of their DNA for further investigation. Blood samples from 180 unaffected French Caucasian individuals were used as controls to estimate the frequency of single nucleotide polymorphism (SNP) and missense variants not available from the HapMap Project. For probands carrying the *FH* mutation, targeted sequencing of *FH* was extended to relatives (58 additional patients).

Genomic rearrangement screening

DNA was extracted from peripheral blood leucocytes according to standard procedures using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, California, USA).Quantitative real-time PCR based on SYBR-green fluorescence technology was used to detect genomic rearrangements (large deletions or duplications of one exon or more). PCR was performed with the QuantiFast SYBR Green PCR Kit (Qiagen) on an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Primers designed for sequencing analysis were used at a concentration of 300 nM. A total of 20 ng DNA was used in a 25 µl reaction volume. The *BRCA1* gene was used as an internal reference. The copy number was determined using the $2-\Delta CT$ method where $\Delta CT = CT_{FH\ amplicon} - CT_{reference\ gene}$.[40]

Sequencing analysis

Mutations in *FH* gene were screened by genomic DNA amplification of each exon and splice junction (primer sequences and PCR conditions available upon request). PCR products from genomic rearrangement screening were first purified using the ExoSAP-IT PCR purification kit (USB) and then sequenced with the Big Dye Terminator v.3.1 kit (Applied Biosystems). Resin Sephadex G50 superfine (Amersham) was used for purification. Sequencing was performed on an ABI3730 automatic DNA sequencer (Applied Biosystems) in 96 well plates. Variants and mutations were identified by visual inspection of the sequence with Seqscape 2.5 software (Applied Biosystems).

Measurement of FH enzyme activity

Lymphoblastoid cell lines (LCL) were generated by Epstein–Barr virus transformation of leucocytes from 20 patients with novel missense or splice mutations and eight wild-type controls. Peripheral blood lymphocytes (PBL) were isolated on Ficoll cushion. LCL and PBL were homogenised in a lyses buffer (50 Mm Tris–HCl pH 7.2 containing 10% Triton X100, 2 mM phenylmethylsulfonyl fluoride, and 0.02% of 2-mercaptoethanol) and subjected to brief sonication. Samples were centrifuged at 10 000 *g* for 20 min at 4°C and the supernatants were used for the enzyme assay. FH enzymatic activity

was measured spectrophotometrically according to standard procedures.[41 42] Briefly, the assay monitors the increase in absorbance at 250 nm due to fumarate production from malate, with a final reaction medium consisting of 50 mM malate and 50 mM phosphate buffer pH 7.8. The FH activity was reported as the amount of fumarate generated per min per mg of protein, or in a ratio to the corresponding citrate synthase activity; final results are then expressed as percentage of control activity.

RESULTS

Mutation analysis

FH genotyping was carried out on germline DNA, and sequencing of the entire gene (coding sequence and exon/intron junctions) revealed 31 different sequence variations in 40/56 families (71.4%) with clinically proven or suspected HLRCC and in 4/23 patients (17.4%) with only PRCCII (2/18 sporadic and 2/5 familial), including the first Chinese origin family described (family F44). The identified mutations included 15 missense, six frameshifts, four nonsense, one deletion/insertion, and five splice site mutations. Country of origin of families, molecular results and references are summarised in table 1.

We completed the *FH* mutational spectrum by exploring large deletion or duplication events and identified one patient with a complete *FH* deletion (proband of family F1, table 1 and figure 1). Twenty-one of the identified mutations have never been described previously (noted 'this report' in table 1), and none of these mutations was found in control DNA samples (0/360 alleles sequenced). These novel mutations included seven missense

Table 1 *FH* germline mutations identified in the study. FH activity in percentage compared to wild-type controls. The activity was determined on lymphoblastoid cell line or (*) on peripheral blood lymphocytes

Family	Origin	Nucleotide change	Amino acid change (HGVS)	Location	FH activity (%)	First description
Families with demonstrative manifestations of HLRCC						
F1	France	c.1-?_1404+?del	p.Met1-?_X468+?del	NA	41.3	6 7 38
F2	France	c.127_128delGA	p.Glu43fs	Exon 2	42*	This report
F3	Portugal	c.138+1_138+10del10	Splice site	Intron 2	50*	This report
F4	France	c.147delT	p.Ile50fs	Exon 3	39.5	This report
F5	Australia	c.172C>T	p.Arg58X	Exon 3	NA	4 6 14 24
F6	France	c.220G>C	p.Ala74Pro	Exon 3	NA	6
F7	New Zealand	c.220G>C	p.Ala74Pro	Exon 3	NA	6
F8	France	c.220G>C	p.Ala74Pro	Exon 3	NA	6
F9	France	c.247_249+1 delGAGGinsA	Splice site	Exon 3	NA	This report
F10	France	c.247_249+1 delGAGGinsA	Splice site	Exon 3	57.2	This report
F11	France	c.250-2A>G	Splice site	Intron 3	49.2	This report
F12	France	c.250-2A>G	Splice site	Intron 3	42.8	This report
F13	France	c.250-2A>G	Splice site	Intron 3	NA	This report
F14	Cambodia	c.298delA	p.Thr100fs	Exon 4	45*	This report
F15	France	c.410A>G	p.His137Arg	Exon 4	44*	6
F16	Morocco	c.503T>C	p.Leu168Pro	Exon 5	52*	This report
F17	Spain	c.568C>T	p.Arg190Cys	Exon 5	42.5	4 39
F18	France	c.568C>T	p.Arg190Cys	Exon 5	NA	4 39
F19	France	c.568C>T	p.Arg190Cys	Exon 5	NA	4 39
F20	France	c.575A>G	p.His192Arg	Exon 5	44	This report
F21	Spain	c.632A>G	p.Gln211Arg	Exon 6	34.6	This report
F22	France	c.666delC	p.Met223fs	Exon 6	49*	This report
F23	Belgium	c.806T>C	p.Phe269Ser	Exon 7	36	This report
F24	France	c.808G>T	p.Glu270X	Exon 7	56.1	This report
F25	France	c.808G>T	p.Glu270X	Exon 7	NA	This report
F26	Canada	c.810delA	p.Ala271fs	Exon 7	NA	This report
F27	France	c.815T>C	p.Leu272Pro	Exon 7	44.4	This report
F28	France	c.821C>T	p.Ala274Val	Exon 7	45.1	Detailed in[43]
F29	Portugal	c.869G>A	p.Cys290Tyr	Exon 7	40	18
F30	France	c.898C>T	p.Arg300X	Exon7	66.7	6
F31	Spain	c.989A>G	p.Asn330Ser	Exon 8	38.7	6
F32	France	c.1028A>G	p.Gln343Arg	Exon 8	47.2*	This report
F33	France	c.1028A>G	p.Gln343Arg	Exon 8	NA	This report
F34	France	c.1060G>A	p.Gly354Arg	Exon 8	46.7	7 20 24 38
F35	France	c.1060G>A	p.Gly354Arg	Exon 8	51.8	7 20 24 38
F36	France	c.1060G>A	p.Gly354Arg	Exon 8	NA	7 20 24 38
F37	Tunisia	c.1108-2A>G	Splice site	Intron 8	50.9	This report
F38	Colombia	c.1121-1123 delTAC	p.Leu374_His375delinsTyr	Exon 9	NA	This report
F39	Canada	c.1265A>G	p.Tyr422Cys	Exon 10	NA	3 14
F40	Israel	c.1371G>A	p.Trp457X	Exon 10	NA	This report
Sporadic and familial type 2 papillary renal cell cancers						
F41	France	c.220G>C	p.Ala74Pro	Exon 3	41.3*	6
F42	Mali	c.426+1G>A	Splice site	Intron 4	58.2*	This report
F43	Morocco	c.988A>G	p.Asn330Asp	Exon 8	46*	This report
F44	China	c.994delA	p.Thr332fs	Exon 8	NA	This report

FH, fumarate hydratase; HLRCC, hereditary leiomyomatosis and renal cell carcinoma; HGVS, Human Genome Variation Society.

(p.Leu168Pro, p.His192Arg, p.Gln211Arg, p.Phe269Ser, p.Leu272Pro, p.Asn330Asp, p.Gln343Arg), two nonsense (p.Glu270X, p.Trp457X), six frameshifts (c.127_128delGA, c.47delT, c.298delA, c.666delC, c.810delA, c.994delA), one insertion-deletion (p.Leu374_His375insdelTyr) and five splice site (138+1_138+10del10, 247_249+1delGAGGinsA, 250−2A>G,426+1G>A, 1108−2A>G). Another mutation (p.Ala274Val) was identified by our group during the study but was published as a case report.[43] Four novel mutations were found in unrelated French families (F9 and F10; F11, F12 and F13; F24 and F25; F32 and F33, respectively) from different areas and there was no evidence of common ancestors. Interestingly, three out of four mutations identified in probands with isolated PRCCII were also novel (table 1).

We then performed an in silico analysis of the putative functional consequences of the missense mutations. As shown in figure 2, all *FH* missense mutations identified in the present study affect residues that have been highly conserved throughout evolution.

Functional characterisation of novel mutations

In order to demonstrate the functional consequences of germline *FH* mutations, we measured the enzymatic activity of endogenous FH in peripheral blood lymphocytes or lymphoblastoid cells derived from patients. We tested 30 patients carrying an *FH* mutation (including 15 unpublished mutations, of which eight are novel missense mutations) and eight samples of the wild-type *FH* gene. Reduction of enzymatic activity by at least 50% was observed for all mutations tested (table 1). We did not notice a major difference in the enzymatic activities of missense mutations (from 34.6% to 54.7% activity compared to a wild-type control, mean activity 45%) and total loss-of-function mutations such as deletions, nonsense mutations, or splice site mutations (from 39.5% to 60.8%, mean activity 50.1%).

Clinical findings

Clinical data could be recovered for 151 relatives belonging to the 44 families with an *FH* mutation and are summarised in table 2. Briefly, cutaneous leiomyomas occurred in 37/44 (84.1%) *FH* mutation positive families that were clinically evaluated by a dermatologist and in 102/151 (67.5%) gene carriers. They were multiple in the 65 patients for which detailed data were available, with the exception of a 76-year-old woman who had only a single leiomyoma without any other clinical manifestation. In eight patients without familial history of MCUL/HLRCC, cutaneous leiomyomas were the only clinical manifestation. Uterine leiomyomas occurred in 32/44 (72.7%) *FH* mutation positive families and in 76/93 (81.7%) affected women and usually were of early onset. In most cases, uterine leiomyomas were multiple, symptomatic and led to hysterectomy before the age of 40 years, but medical records were obtained for only a few patients.

Renal tumours occurred in 15/44 (34%) of families with *FH* mutation and in 27/151 (17.9%) of affected members. There were 19 men and eight women affected with RCC and the age at diagnosis of RCC, known for 21 patients, was an average of 43 years (range 28–70). In all these patients, RCC was revealed by clinical symptoms (mainly haematuria, abdominal, lumbar or bony pains) and to date no renal tumour was detected in other gene carriers. Pathological analysis of RCC demonstrated 16 PRCCII (including one sarcomatoid), two collecting duct RCC,

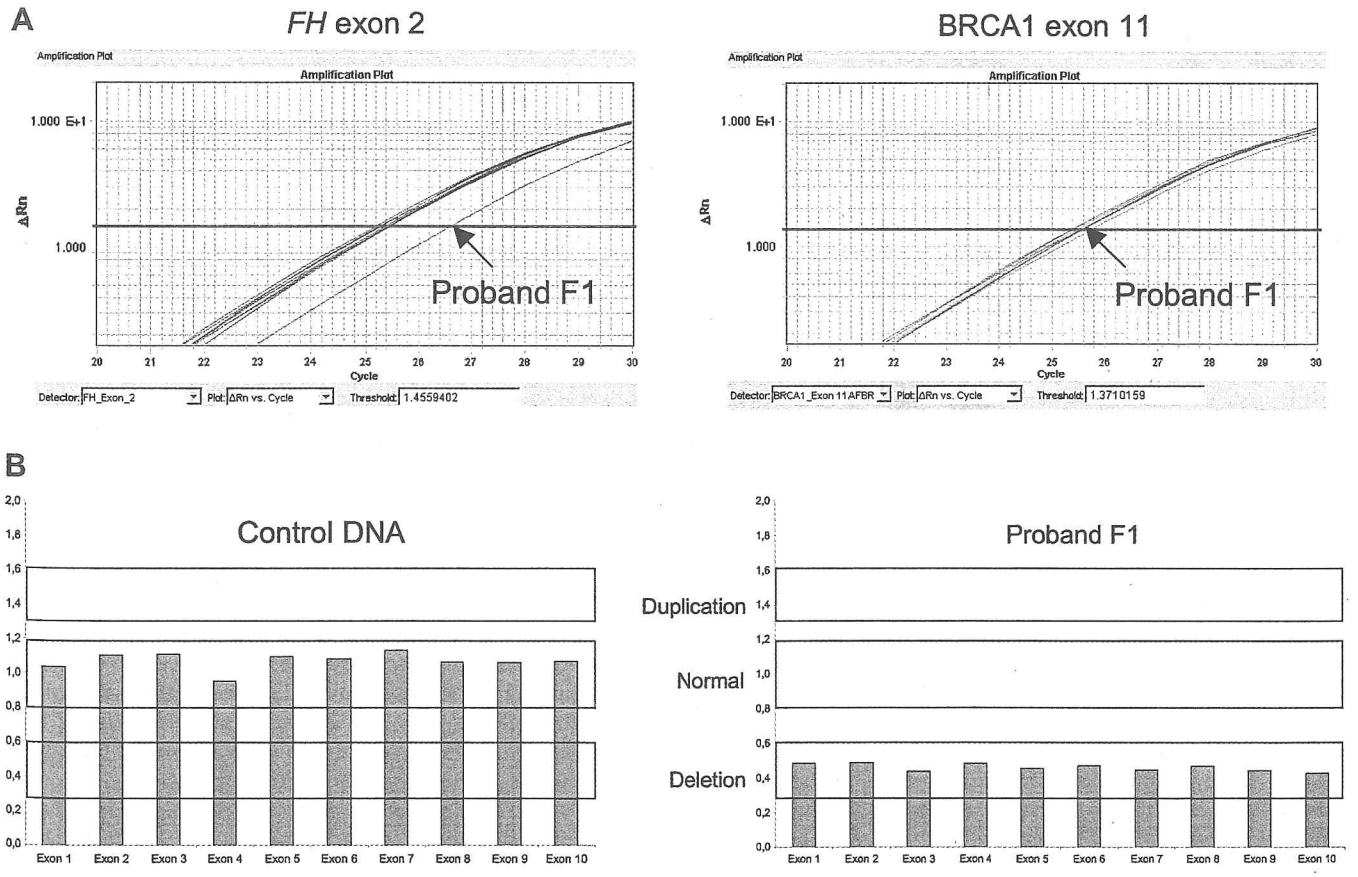

Figure 1 Genomic rearrangement study. (A) Amplification plots obtained by real-time PCR for exon 2 of *FH* and for the control gene (exon 11 of *BRCA1*) in proband of family F1. (B) Gene copy number for all *FH* exons detected in control DNA and in patient's DNA.

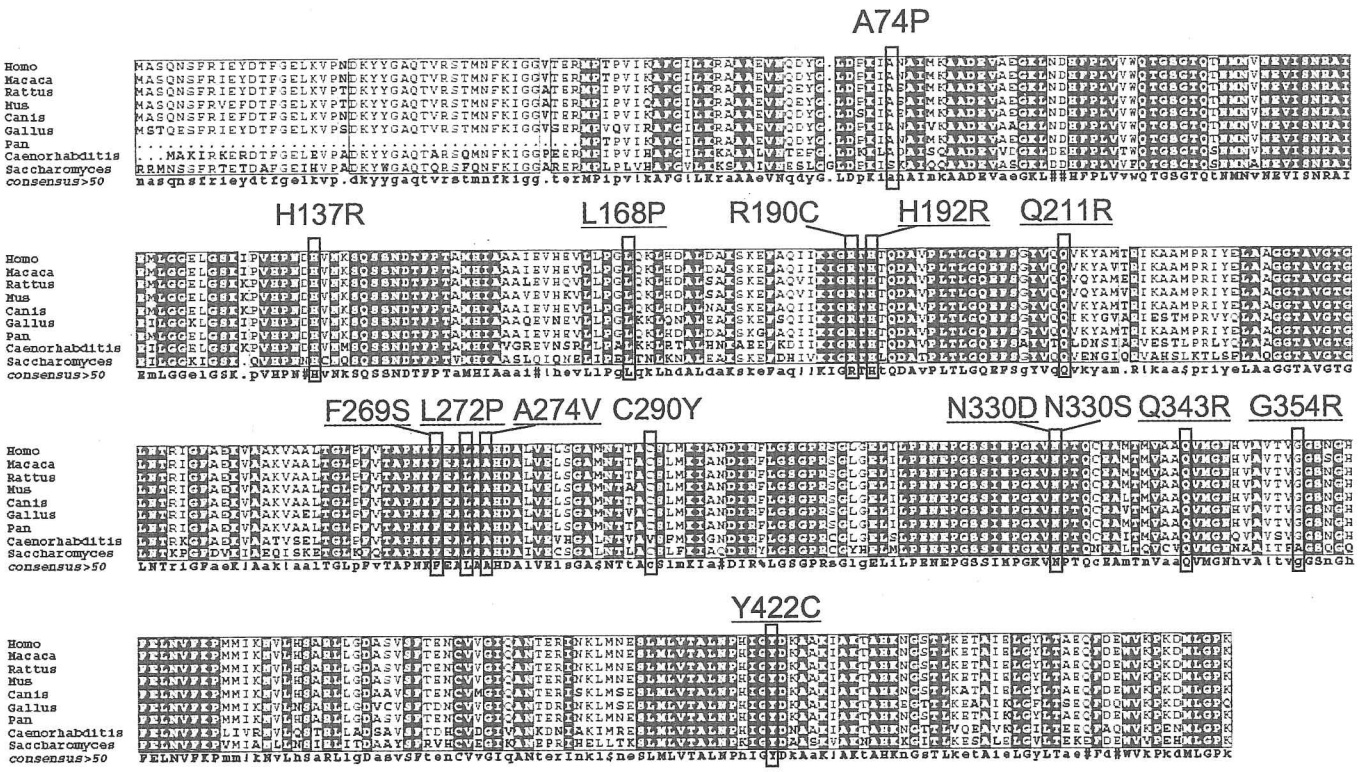

Figure 2 Alignment of fumarate hydratase (FH) protein across 10 species using the Multalin interface. (http://bioinfo.genopole-toulouse.prd.fr/multalin/). From top to bottom: *Homo sapiens*, macaque, rat, mouse, dog, chicken, chimpanzee, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, and *Escherichia coli*. High consensus residues (90% conservation) are represented as white font on a red background; low consensus residues (50% conservation) are in red font; non-conserved residues are in black font. Missense mutations identified in this study are indicated, and underlines highlight novel mutations.

and one sarcomatoid clear cell RCC. Data were missing for the eight last patients. Twenty patients (74.1%) died because of metastatic RCC and the mean age, known for 14 patients, was 44 years (range 17–66). In four families there was no other clinical manifestation including two families with four cases of RCC each and two index cases presenting as sporadic PRCCII. In addition, one patient (family F37) aged 31 years had bilateral atypical renal cysts on CT scan but no tumour.

The clinical history of the patient with p.Ala274Val mutation was detailed in a very recent paper because of the unique association of cutaneous leiomyomatosis with cutis verticis gyrata, disseminated collagenoma, and Charcot–Marie–Tooth's disease.[43]

There was no clear genotype–phenotype correlation specially regarding the occurrence of renal tumours. On the other hand, we observed an intrafamilial phenotypic heterogeneity as illustrated in figure 3. Moreover, genetic testing was positive in five asymptomatic (20–65 years) relatives in which detailed clinical investigations demonstrated no manifestation (figure 3).

DISCUSSION

The present study reports the identification and analysis of *FH* mutations, including 21 novel, in 40 families with MCUL/HLRCC and, for the first time, in four patients with isolated PRCCII. The mutations identified in our series include, at the protein level, 23 (52.3%) missense mutations, seven (15.9%) frameshifts, five (11.4%) nonsense mutations, one (2.3%) microdeletion/insertion, and eight (18.2%) sequence variations affecting splice sites. These results are comparable to the *FH* mutation database; missense mutations are the most common type of germline *FH* mutation in MCUL/HLRCC families (64%), followed by frameshifts (14.6%) and nonsense (12.5%) mutations.[21] Additionally, we have doubled the number of known splice site mutations (identified in eight families (18.2%) in the present study versus seven (4.9%) previously described worldwide), emphasising the importance of careful analysis of non-coding sequences surrounding splice sites. We found only one family with a large deletion, thus confirming that germline copy loss of *FH* is a rare genetic event (only four large deletions and one deletion of exon 1 in 144 HLRCC families described).[6]

In order to evaluate the potential deleterious effect of novel nucleotide variations identified in the *FH* gene, we measured the enzymatic activity of endogenous FH in lymphoblastoid cells derived from patients. Reduction of at least 50% of the enzymatic activity was observed for all mutations tested, thus supporting a model of loss of function and haploinsufficiency. Mutant FH proteins have been postulated to exert a dominant negative effect based on FH enzymatic activity[4 6 7]; this hypothesis is supported by in vitro overexpression experiments in which the R190H *FH* mutant induced 60% inhibition of the endogenous enzymatic activity.[44] As the active FH enzyme is a homotetramer, it is theoretically possible that missense mutations may affect FH activity more drastically than nonsense mutations; these mutations could disrupt the formation of nearly all wild-type homotetramers, leaving only one in 16 (1/24) tetramers composed of exclusively wild-type subunits. Therefore, a missense variant behaving as a strong dominant negative could dramatically reduce enzymatic activity of heterotetrameric FH. Such molecular behaviour has never been described, and we did not notice a major difference in the

Table 2 Clinical manifestations observed in patients with identified *FH* mutation

Family	Mutation	Patients	Cutaneous leiomyomas	Uterine leiomyomas*	Renal cell carcinoma (RCC)			
					Number	Sex	Age	RCC type
Families with demonstrative manifestations of HLRCC								
F1	c.1-?_1404+?del	5	5	1/2	0			
F2	c.127_128delGA	1	1	0/0	0			
F3	c.138+1_138+10del10	2	2	2/2	0			
F4	c.147delT	1	1	0/0	0			
F5	c.172C>T	4	3	2/4	0			
F6	c.220G>C	2	2	1/1	0			
F7	c.220G>C	1	1	NA	0			
F8	c.220G>C	1	1	0/1	0			
F9	c.247_249+1 delGAGGinsA	1	1	1/1	1	F	43	PRCCII
F10	c.247_249+1 delGAGGinsA	9	6	3/4	2	M	35	CD RCC
						M	52	PRCCII
F11	c.250-2A>G	3	2	1/1	1	M	34	PRCCII
F12	c.250-2A>G	1	1	1/1	0			
F13	c.250-2A>G	2	1	2/2	0			
F14	c.298delA	2	1	2/2	0			
F15	c.410A>G	3	2	2/2	2	M	65	NA
						F	30	SR-PRCCII
F16	c.503T>C	4	4	2/2	0			
F17	c.568C>T	7	3	7/7	0			
F18	c.568C>T	5	4	1/2	1	M	NA	NA
F19	c.568C>T	2	1	2/2	0			
F20	c.575A>G	3	3	2/2	0			
F21	c.632A>G	2	1	2/2	0			
F22	c.666delC	4	1	3/4	0			
F23	c.806T>C	1	1	1/1	0			
F24	c.808G>T	3	2	0/1	0			
F25	c.808G>T	1	1	0/1	0			
F26	c.810delA	3	2	2/2	1	M	49	NA
F27	c.815T>C	4	3	3/3	0			
F28	c.821C>T	6	4	2/3	0			
F29	c.869G>A	4	1	4/4	0			
F30	c.898C>T	6	3	6/6	0			
F31	c.989A>G	4	3	2/2	0			
F32	c.1028A>G	2	2	1/1	0			
F33	c.1028A>G	6	5	3/3	1	F	34	PRCCII
F34	c.1060G>A	7	5	3/4	3	M	59	PRCCII
						M	57	PRCCII
						M	NA	NA
F35	c.1060G>A	4	3	3/4	2	F	50	SR RCC
						F	52	PRCCII
F36	c.1060G>A	5	5	0/2	0			
F37	c.1108-2A>G	1	1	0/0	0			
F38	c.1121-1123 delAC	5	NA	3/4	2	M	40	NA
						F	28	PRCCII
F39	c.1265A>G	1	1	1/1	0			
F40	c.1371G>A	17	16	5/5	1	M	36	CD RCC
Sporadic or familial type 2 papillary renal cell cancers								
F41	c.220G>C	1	0	0	1	M	34	Sp PRCCII
F42	c.426+1G>A	1	0	0	1	M	37	Sp PRCCII
F43	c.988A>G	4	0	0	4	M	31	PRCCII
						M	40	PRCCII
						F	59	PRCCII
						M	70	PRCCII
F44	c.994delA	4	0	0/1	4	M	17	PRCCII
						M	34	PRCCII
						M	45	PRCCII
						F	69	PRCCII

*In women.
CD, collecting duct; F, female; M, male; NA, data not available; PRCCII, type 2 papillary renal cell cancers; Sp, sporadic; SR, sarcomatoid.

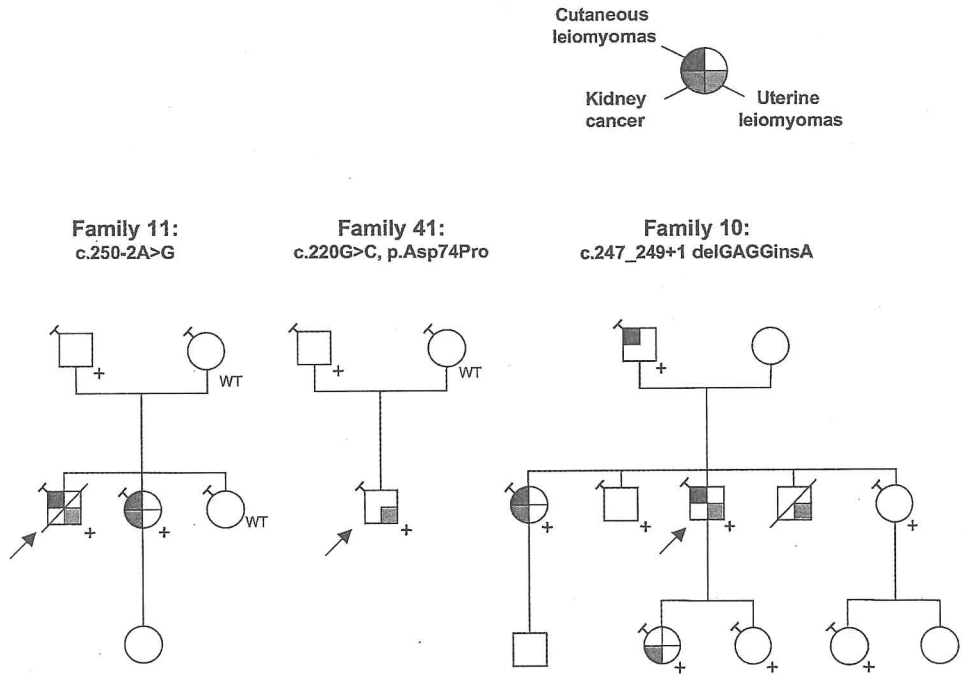

Figure 3 Pedigrees of representative families with hereditary leiomyomatosis and renal cell carcinoma illustrating phenotypic heterogeneity. Solid symbols represent affected family members, and a slash indicate deceased family members. Probands are identified by an arrow. The result of the genetic testing is indicated: + (mutated) or WT (wild type).

enzymatic activities of missense mutations versus total loss of function mutations. Therefore, we conclude that the missense allele products do not exert a strong dominant negative effect in vivo.

Genotype–phenotype relationships between the *FH* mutational spectrum and HLRCC manifestations have been previously proposed. For example, p.Arg58X *FH* mutations have been associated with a high frequency of kidney cancers,[4] but the carrier of this mutation in our series did not develop RCC by the age of 62 years. The p.Gly354Arg mutation has been reported to predispose patients exclusively to fibroids,[7] but we found this mutation in families that displayed various phenotypes of skin lesions and renal cancers. In addition, several mutations that have been described in families with RCCs (p.Arg190Cys, p.Arg300X, p.Asn330Ser) or without RCCs (p.His137Arg) were associated with the opposite phenotype in our study. Phenotype heterogeneity is also observed within families (figure 3). Therefore, there is to date no convincing evidence of a relationship between the type of *FH* mutation and the resulting disease phenotype.

We observed *FH* mutations in only 71.4% (40/56) of the MCUL/HLRCC families included in our study, a lower ratio compared with previous reports. This difference may be due to the recruitment of probands who not fulfil all clinical criteria of the HLRCC disease (especially patients with isolated cutaneous leiomyomas or patients with uterine leiomyomas and RCC). On the other hand, these broad inclusion criteria allowed us to identify seven new families with HLRCC that would have not been detected otherwise. Indeed, the recruitment of patients by urologists allowed us to revise the percentage of families with RCC to 40% from the previously reported 20% (29/144 families). Concerning the percentage of all individuals with an *FH* mutation, we observed an RCC incidence of 17.9%, a figure similar to the 10–16% incidence previously described.[3]

In addition, we describe *FH* germline mutations in two families with a history of PRCCII only (no documented history of leiomyomatosis) and, for the fist time, in two patients with apparent sporadic PRCCII. The p.Ala74Pro mutation (family F41) has been previously reported in the context of HLRCC without RCC[6]; however the splice site mutation c.426+1G>A (family F42), the p.Thr332fs mutation (family F44), and the p.Asn330Asp mutation (family F43) are novel. Interestingly, the unaffected father of family F44 proband's can be considered an obligate carrier, knowing that his mother and two of his brothers developed PRCCII. Consequently, we expanded the *FH* genotyping to relatives of the patients diagnosed with an *FH* mutation (58 more family members). As a result, we identified five patients with *FH* germline mutations who lacked any manifestation of the disease, similar to families described by Wei *et al*.[4]

This question of incomplete penetrance is also raised for the parents of child carriers of homozygous/compound heterozygous *FH* germline mutations, who are obligate heterozygous *FH* mutation carriers. These observations suggest low penetrance but need confirmation by analysing a larger family set. As accumulation of fumarate subsequent to FH activity loss may contribute to the pathology,[33] incomplete penetrance could thus be due to individual variation in FH enzymatic activity in relation with other host factors. Therefore, we compared the FH enzymatic activity of cells from patients of the same family exhibiting severe phenotypes or no to very mild phenotypes. We obtained comparable results with the same loss of FH activity in the asymptomatic patients (supplementary table 2).

Taken together, these results demonstrate that the phenotypic spectrum of *FH* carriers is broader than expected, extending from asymptomatic to severe disease with multiple tumours including RCC. Additional genetic or environmental modifying factors may play an important role in the development of the disease. Fine mapping and haplotype analysis surrounding the *FH* gene failed to identify a genetic modifier for RCC risk in HLRCC families (probands of families F11 and F34),[24] and a differential transcriptional study of asymptomatic versus symptomatic patients showed no significant differences (families F10, F11, and F41, data not shown). It could be interesting to perform a metabolomic study in patients who are minimally affected clinically to investigate whether they have developed alternative pathways to compensate for the *FH* mutation. Hence, due to the incomplete penetrance of HLRCC, genetic

testing of *FH* should be conducted more widely, and should be applied to patients with apparent sporadic PRCCII when the patient is <40 years old at diagnosis or when the histology is characteristic of HLRCC as recently defined by Merino *et al* (large nucleus with proeminent oriangiophilic or eosinophilic nucleolus surrounded by a clear halo).[25] This expanded testing regimen could allow the discovery of previously undiagnosed HLRCC families, leading to appropriate clinical management including dermatological surveillance and gynaecological examination in women at potential risk for early hysterectomy. Indeed, careful skin examination of the proband of family F41 (affected with an apparent sporadic PRCCII) was performed after *FH* mutation identification and revealed a small cutaneous leiomyoma.

In addition to the potential implications for presymptomatic diagnosis, identification of patients with *FH* germline mutations could also be critical for the determination of the appropriate treatment of advanced PRCCII. Indeed, as hereditary PRCCIIs are characterised by HIF overexpression and activation of angiogenesis,[32] it would be useful to explore the efficacy of anti-angiogenic drugs in patients affected by such tumours. Recently, prolonged progression-free survival times were reported in 2/12 patients with apparent sporadic PRCCII treated by sunitinib, a novel tyrosine kinase inhibitor.[45] Further investigation is needed, but preliminary observation showed that one of these two patients presented with cutaneous leiomyomas and is certainly a carrier of a germline *FH* mutation. Thus, it would be of great interest to explore the potential predictive role of *FH* mutations in therapy response.

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